



Inorganic mercury modifies Ca^{2+} signals, triggers apoptosis and potentiates NMDA toxicity in cerebellar granule neurons

Anna D. Rossi^{1,2}, Barbara Viviani¹, Boris Zhivotovsky¹,
Luigi Manzo², Sten Orrenius¹, Marie Vahter¹ and
Pierluigi Nicotera^{1,3,4}

- ¹ Institute of Environmental Medicine, Division of Toxicology, Karolinska Institutet, Stockholm, Sweden
² Clinica del Lavoro Medical Center, University of Pavia, Italy and
³ Faculty of Biology, Chair of Molecular Toxicology, University of Konstanz, Germany
⁴ corresponding author: Prof. Pierluigi Nicotera, Faculty of Biology, Chair of Molecular Toxicology, Box 5560 X911, University of Konstanz, D-78434 Konstanz, Germany. tel: + 49 7531 8840 35; fax: + 49 7531 8840 33; e-mail: Pierluigi.Nicotera@uni-konstanz.de

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Abstract

Hg^{2+} (0.1 μM - 0.5 μM) modified the Ca^{2+} signals elicited by either KCl or the glutamate-receptor agonist, N-methyl-D-aspartate (NMDA), in cerebellar granule cells (CGCs). Hg^{2+} enhanced the intracellular Ca^{2+} transient elicited by high K^+ and prevented a complete recovery of the resting intracellular Ca^{2+} concentration ($[\text{Ca}^{2+}]_i$) after either KCl or NMDA stimulation. Higher Hg^{2+} concentrations (up to 1 μM) increased $[\text{Ca}^{2+}]_i$ directly. Following the short-term exposure to Hg^{2+} , CGCs underwent apoptosis, which was identified by the cleavage of DNA into large (700–50 kbp) and oligonucleosomal DNA fragments, and by the appearance of typical apoptotic nuclei. Combined treatment with 0.1–0.3 μM Hg^{2+} and a sublethal NMDA concentration (50 μM) potentiated DNA fragmentation and apoptotic cell death. When the exposure to Hg^{2+} was carried out in Ca^{2+} -free media or in the presence of Ca^{2+} channel blockers (L-type or NMDA-R antagonists), the effects on signalling and apoptosis were prevented. Our results suggest that very low Hg^{2+} concentrations can trigger apoptosis in CGCs by facilitating Ca^{2+} entry through membrane channels.

Keywords: apoptosis, calcium, excitotoxicity, L-type channels

Abbreviations: cerebellar granule cells, CGCs; (ethylenediamine) tetra acetic acid disodium salt, EDTA; acetoxymethyl ester of fura 2, fura 2AM; fetal calf serum, FCS; field-inversion gel electrophoresis, FIGE; kilobase pair, kbp; dibenzocyclohepteneiminemaleate, MK-801; 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyltetrazolium bromide, MTT; nerve growth factor, NGF; N-methyl-D-aspartate, NMDA; tris-borate/EDTA electrophoresis buffer, TBE

Introduction

Over the past several years, much attention has focused on the role of perturbations of Ca^{2+} signalling and oxidative stress in the development of neurotoxic disorders (Coyle and Puttfarcken, 1993). Mild oxidation can modulate agonist/receptor interaction, neurotransmitter or growth factor stimulation and affect Ca^{2+} channel conductance (Nicotera *et al*, 1992; Rossi *et al*, 1993). Marginally higher pro-oxidant levels can cause cell death by overstimulation of physiological signalling or by recruitment of cytotoxic reactions (Nicotera *et al*, 1992; Dypbukt *et al*, 1994). Amplification of agonist-stimulated Ca^{2+} transients in cells previously exposed to mild oxidation can also potentiate physiological processes such as cell secretory activity or differentiation (Viviani *et al*, 1996; Rossi *et al*, 1993). Again, when the duration and extent of the Ca^{2+} increase exceed the cell regulatory capacity, death may ensue (Choi, 1989; Nicotera *et al*, 1994). It has also become clear that increasing the intensity of a given insult will result in the progressive recruitment of multiple cytotoxic mechanisms shifting the mode of cell death from apoptosis to necrosis (Dypbukt *et al*, 1994; Bonfoco *et al*, 1995). Indeed, most studies on apoptosis, have emphasized the concept that interference with signalling systems can be sufficient to stimulate or repress cells' own death program.

Cellular responses to endogenous or environmental toxicants are also quite dependent on the degree of differentiation. Thus, cell death may occur in dividing cells because of unbalanced mitogenic stimulation, whereas the same condition may not cause cytotoxicity in differentiated cells (Johnson and Deckwert, 1994). Studies in our laboratory have shown that metals such as mercury and tin can alter neuronal differentiation (Rossi *et al*, 1993), affect neurotransmitter production (Viviani *et al*, 1996) and ultimately activate apoptosis in cell lines (Viviani *et al*, 1995). In particular, inorganic mercury caused alterations in the conductance of L-type Ca^{2+} channels, potentiated Ca^{2+} signals elicited by depolarization or agonist stimulation, and enhanced NGF-induced differentiation in PC12 cells (Rossi *et al*, 1993).

In view of our previous findings, this study was designed to investigate whether exposure to Hg^{2+} concentrations that stimulate PC12 cell differentiation would have different implications for post-mitotic neurons. As model system, we selected primary cultures of rat cerebellar granule cells (CGCs). After a few days in culture, CGCs, the most abundant neuronal population in the cerebellum (D'Mello *et al*, 1993), express glutamate receptors of the NMDA subtype and can selectively release L-glutamate and L-aspartate upon depolarization (Cull-Candy *et al*, 1988). These characteristics enabled us to study the interference of Hg^{2+} with Ca^{2+} signalling elicited by either high K^+ or

NMDA and to investigate the implication of this interaction for cell survival.

Results

Effect of inorganic mercury on K⁺-induced depolarization

Perfusion of CGC with a buffer containing 50 mM KCl produced a rapid increase in [Ca²⁺]_i. As illustrated in Figure 1A, the [Ca²⁺]_i rapidly reached a peak and, subsequently, a

plateau level. After washing, normal Ca²⁺ resting levels were reinstated. A second KCl addition triggered a similar Ca²⁺ response in 90% of the cells, whereas in the remaining 10% the rise in [Ca²⁺]_i was smaller than that elicited by the first depolarization, in accordance with previous observations (Connor *et al*, 1987). When Hg²⁺ (0.1 μM) was added after the first stimulation, the second Ca²⁺ peak increased in the majority of the cells, while the plateau was unaffected (Figure 1B). The net Ca²⁺ rise (eg, net elevation from the resting level) elicited by high K⁺ in the absence of Hg²⁺ was 249 ± 62 nM. In the presence of 0.1 μM Hg²⁺, the Ca²⁺

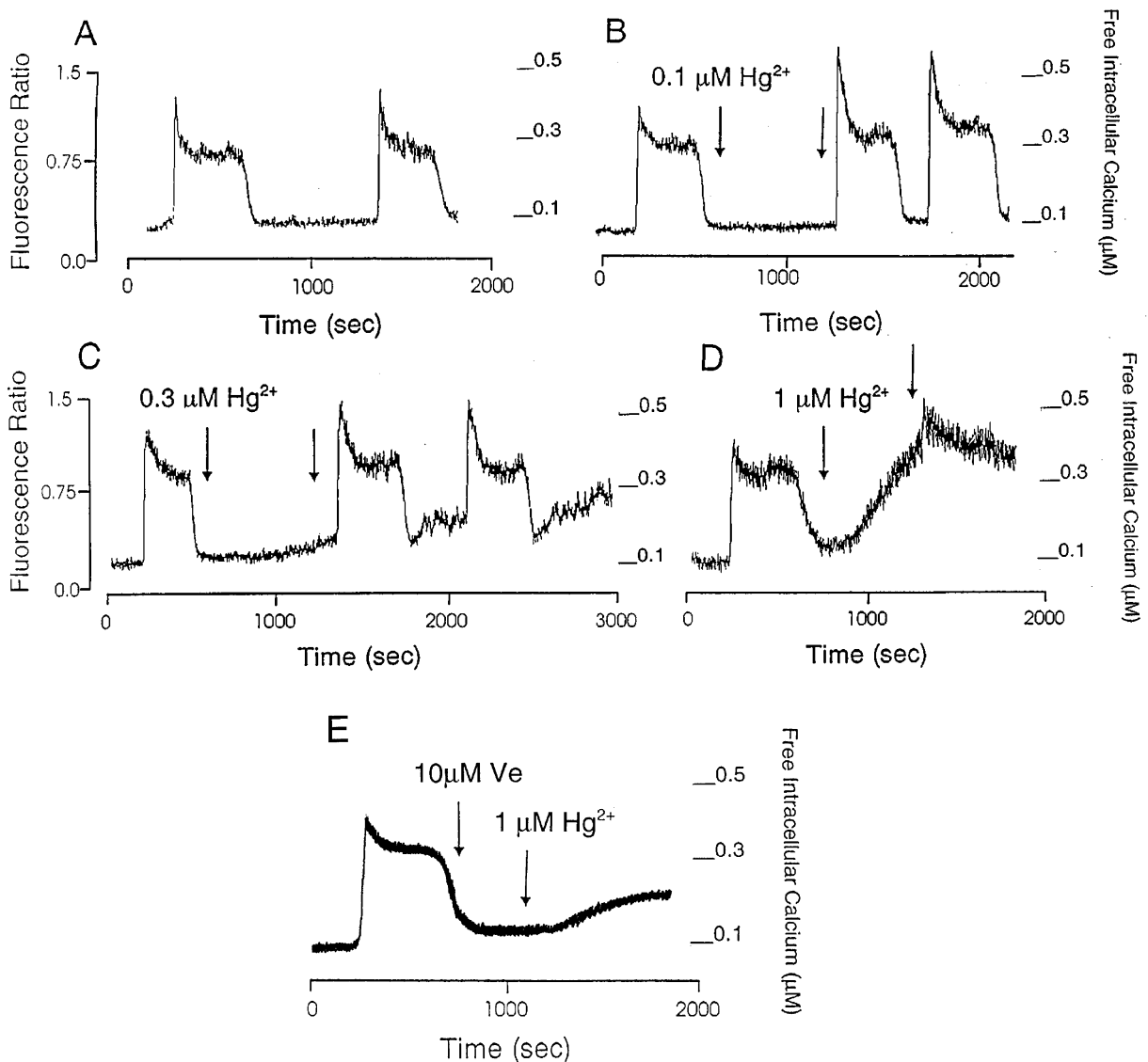


Figure 1 Effects of Hg²⁺ on the resting [Ca²⁺]_i and KCl-elicited Ca²⁺ signals in CGCs. Cells grown on glass coverslips were loaded with fura 2 AM as described in Materials and Methods and placed under a Zeiss Axiovert microscope connected to a Spex Fluorolog Ca²⁺ analysis system. Individual cells or small group of cells, up to five were imaged in each coverslip (100 cells were examined in four separate experiments) Trace **A**: control cells were stimulated with 50 mM KCl, washed and stimulated again 10 min after [Ca²⁺]_i had returned to basal level. Trace **B**: CGCs were initially stimulated with 50 mM KCl as above, then they were washed and reincubated for 10 min with 0.1 μM Hg²⁺. Thereafter cells were stimulated twice with KCl. Trace **C**: cells were treated as in trace B, but Hg²⁺ concentration was 300 nM. After Hg²⁺ CGCs were stimulated twice with KCl. Trace **D**: following KCl stimulation and subsequent washing, cells were exposed for 10 min to 1 μM Hg²⁺ and again stimulated with KCl which gave rise to a small peak superimposed to the Ca²⁺ overload elicited by Hg²⁺. Trace **E**: cells were initially treated as in trace A, then washed in nominally Ca²⁺ free medium and reincubated in the same medium supplemented with 10 μM verapamil (VE). Hg²⁺ was then added and the [Ca²⁺]_i measured for the next 15 min.

elevation was 443 ± 54 nM ($n=100$ cells; $p < 0.05$ by Student's *t* test).

If depolarization was induced in cells pretreated with a slightly higher Hg^{2+} concentration ($0.3 \mu M$) again the $[Ca^{2+}]_i$ increased. However, in this case the resting Ca^{2+} levels were not completely restored (Figure 1C). Higher Hg^{2+} concentrations ($1-2 \mu M$) directly elicited a sustained Ca^{2+} increase. In addition, following the exposure to high Hg^{2+} concentrations the Ca^{2+} transient elicited by KCl was inhibited (Figure 1D). Addition of verapamil, a L-type Ca^{2+} channel blocker, after K^+ depolarization, blocked Hg^{2+} -induced increase in $[Ca^{2+}]_i$ (Figure 1E).

Effect of inorganic mercury on NMDA-induced Ca^{2+} responses

N-methyl-D-aspartate (NMDA) receptors in CGCs are accountable for most of the Ca^{2+} increase elicited by glutamate, as shown by the observation that dibenzocyclohepteneiminemaleate (MK-801), a selective NMDA antagonist, virtually blocks Ca^{2+} entry elicited by this excitatory amino acid (Eimerl and Schramm, 1992). To relieve the NMDA receptor from the block exerted by Mg^{2+} and to sensitize the receptor to NMDA stimulation, exposures were

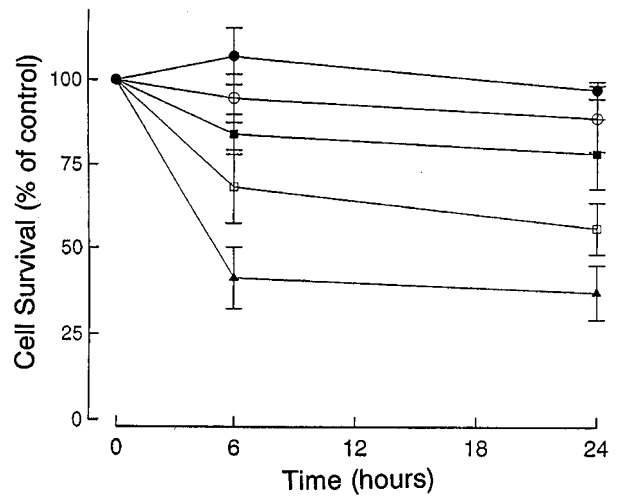


Figure 3 Cell killing induced by Hg^{2+} in CGCs. Cells were incubated for 10 min with different concentrations of Hg^{2+} and the MTT assay was performed as described in Materials and Methods. Symbols: ●, $0.1 \mu M$ Hg^{2+} ; ○, $0.3 \mu M$ Hg^{2+} ; ■, $0.5 \mu M$ Hg^{2+} ; □, $1 \mu M$ Hg^{2+} ; ▲, $2 \mu M$ Hg^{2+} . Results are expressed as percent of formazan formation as measured in control cells. Each point represents the mean \pm s.e. of triplicate samples obtained from four separate experiments.

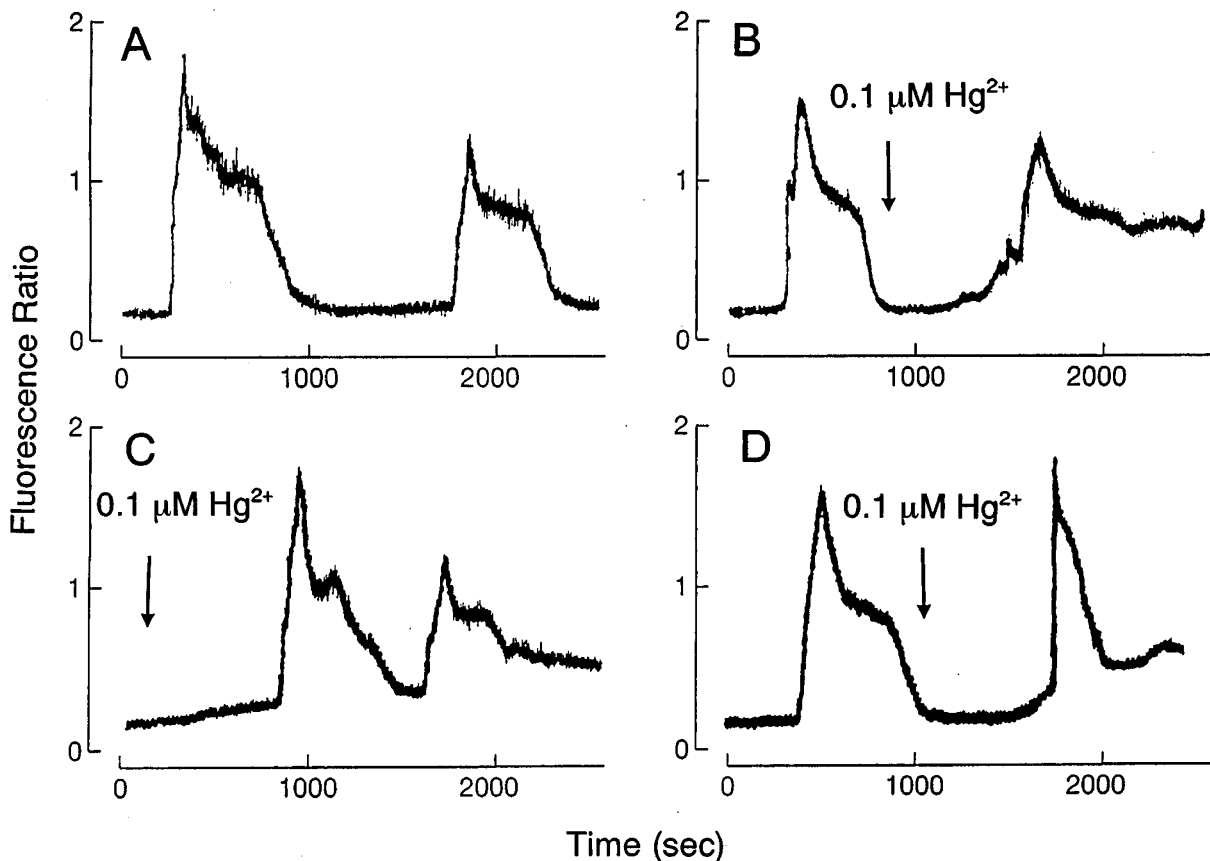


Figure 2 Effect of Hg^{2+} on Ca^{2+} signals elicited by NMDA in CGCs. $[Ca^{2+}]_i$ was measured as described in Figure 1. Trace A: cells were stimulated with $100 \mu M$ NMDA washed and reincubated for 10 min and then stimulated a second time. Trace B: after the first stimulation with $100 \mu M$ NMDA, cells were incubated for 10 min with $0.1 \mu M$ Hg^{2+} and then stimulated with NMDA a second time. Trace C: cells were initially incubated for 10 min with $0.1 \mu M$ Hg^{2+} and stimulated twice with $100 \mu M$ NMDA. Trace D: cells were treated like in trace B. A spontaneous Ca^{2+} spike was observed after incubation with $0.1 \mu M$ Hg^{2+} . The subsequent stimulation with NMDA (indicated by the arrow) did not elicit a new Ca^{2+} spike. Traces represent one experiment typical of four where at least 100 cells were analyzed.

performed in a nominally Mg^{2+} -free medium, supplemented with 10 μM glycine (Johnson and Asher, 1987; Zorumski and Thio, 1992; Scatton, 1993). We selected NMDA concentrations (50–100 μM) that elicited a maximal transient $[Ca^{2+}]_i$ elevation without promoting neuronal death. After addition of 100 μM NMDA, the $[Ca^{2+}]_i$ rapidly increased, reached a peak, declined to a plateau level and, finally, returned to resting level after washing. A second NMDA stimulation caused a qualitatively similar increase in $[Ca^{2+}]_i$, which was however, less pronounced (Figure 2A). When cells were stimulated with NMDA, treated for 10 min with 0.1 μM Hg^{2+} and then washed, the $[Ca^{2+}]_i$ did not return to the resting level (Figure 2B). Treatment with these Hg^{2+} concentrations in the absence of NMDA did not affect the resting $[Ca^{2+}]_i$. When cells were

pretreated with Hg^{2+} before the first NMDA addition, the Ca^{2+} response to NMDA was not changed. However, the recovery after subsequent stimulations was still incomplete (Figure 2C). Noticeably, in some experiments when Hg^{2+} was added after NMDA, spontaneous, random Ca^{2+} spikes were sometimes observed (Figure 2D).

Exposure to Hg^{2+} induces CGC death

Exposure to Hg^{2+} caused cell killing in a time- and concentration-dependent fashion as assessed by the decreased capacity of CGC to sequester and metabolize MTT (Figure 3). With low Hg^{2+} concentrations (i.e. 0.1–0.5 μM) impairment of MTT metabolism was minimal,

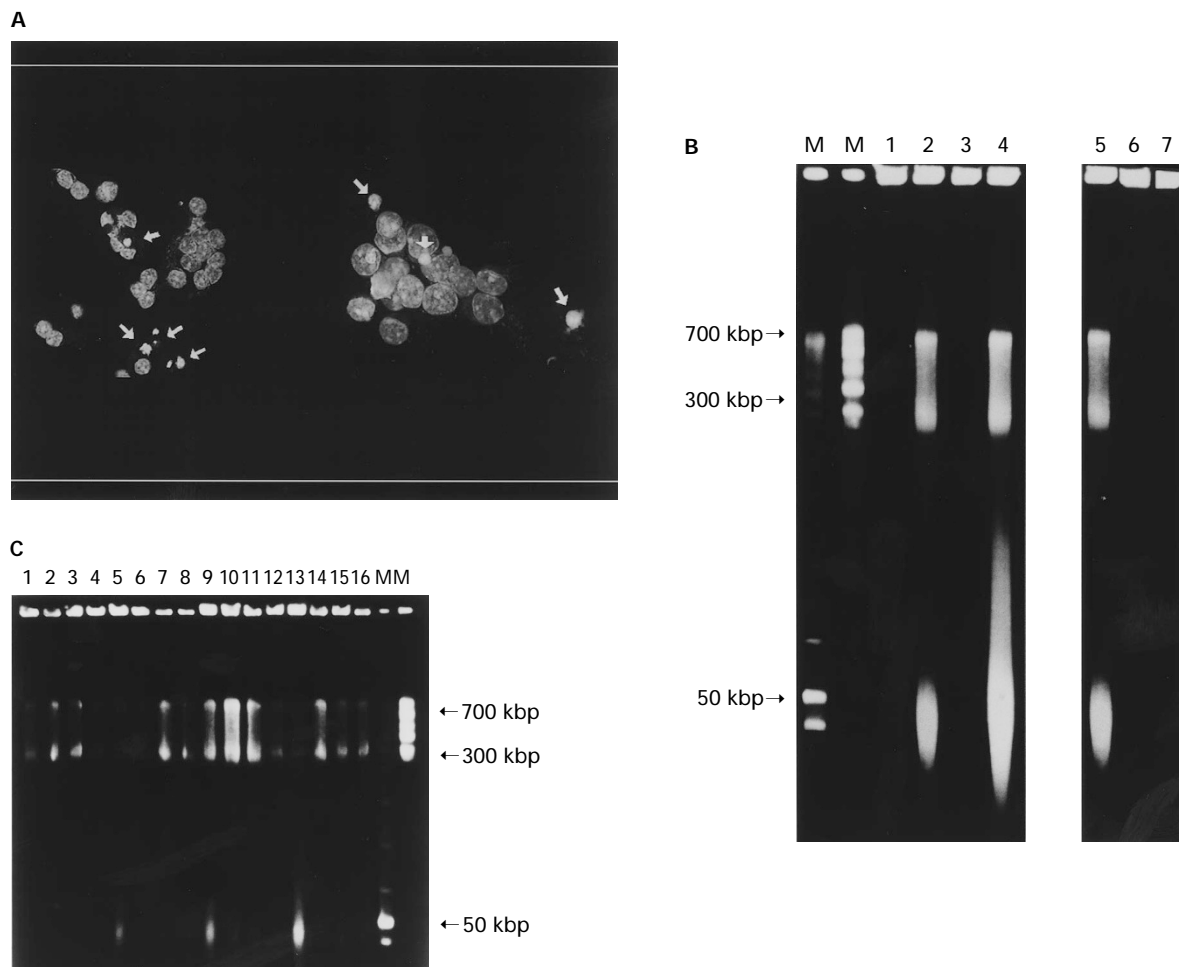


Figure 4 Apoptotic body formation and oligonucleosomal chromatin fragmentation induced by Hg^{2+} . (A) CGCs were treated with 0.5 μM Hg^{2+} for 10 min. Cells were then washed and reincubated in culture medium. After 24 h, formation of apoptotic nuclei was detected by confocal microscopy. The arrows indicate apoptotic nuclei typically pycnotic with condensed chromatin. On the right side of the image a 1.5 zoom factor has been used to better show the difference in size and morphology between apparently normal nuclei and those undergone apoptotic condensation. At least 10 fields, or more than 400 cells, were imaged in each sample from three separate experiments. (B) CGCs were treated with 0.5 μM Hg^{2+} alone or in conjunction with 50 μM NMDA, or under Ca^{2+} -free conditions followed by replacement of the medium with one containing 10 μM verapamil. Large DNA fragments were detected by field inverted pulsed gel electrophoresis (FIGE). M: DNA markers; lane 1: control, lane 2: 0.5 μM Hg^{2+} , lane 3: 50 μM NMDA; lane 4: 0.5 μM Hg^{2+} + 50 μM NMDA, lane 5: 0.5 μM Hg^{2+} ; lane 6: control cells bathed in Ca^{2+} -free medium + 10 μM verapamil; lane 7: 0.5 μM Hg^{2+} , in Ca^{2+} -free medium plus 10 μM verapamil. (C) Control CGCs lanes 1–4, or exposed to Hg^{2+} 0.5 μM lanes 5–8, or to NMDA (500 μM) lanes 9–12, or to Hg^{2+} (0.5 μM) plus NMDA (500 μM) lanes 13–16. Pretreatment with 1 μM MK-801 is shown in lanes 2, 6, 10, 14; with 0.5 μM nifedipine in lanes 3, 7, 11, 15; with 30 μM nicardipine in lanes 4, 8, 12, 16. M indicates the two sets of markers described in Material and Methods. Fragments of 50 kbp are visible after treatment with Hg^{2+} (lane 5), NMDA (lane 9) or the combination of Hg^{2+} plus NMDA (lane 13). B and C show one experiment typical of three.

whereas 1 and 2 μM Hg^{2+} inhibited MTT metabolism within the first 6 h. No additional loss of MTT metabolism was detected during the following 24 h.

However, between 6 and 24 h neurons progressively underwent apoptosis as judged by morphological and biochemical criteria. Hg^{2+} induced the formation of apoptotic nuclei as shown by fluorescence microscopy of ethidium homodimer stained cells (Figure 4A). Further, bands corresponding to 700, 300 and 50 kbp DNA fragments, (Zhivotovsky *et al*, 1994) were detected by FIGE (Figure 4B, and C). Fragments of 50 kbp appeared in concomitance with the detection of typical apoptotic nuclei. Treatment with Hg^{2+} in Ca^{2+} -free buffers and subsequent incubation with verapamil to prevent secondary Ca^{2+} entry after medium replacement prevented the accumulation of the 50 kbp DNA fragments (Figure 4B lanes 5–7). Similarly, when CGCs were exposed in a medium containing normal $[\text{Ca}^{2+}]$, but supplemented with one of the channel blockers MK-801 (NMDA-R channels) or nifedipine and nicardipine (L-type channels), the formation of the 50 kbp fragments was also prevented (Figure 4C, lanes 5–8). The formation of smaller, oligonucleosomal-sized fragments (Arends *et al*, 1990) was also detected in the $15,000 \times g$ supernatant fraction of CGC lysates using monoclonal antibodies directed against DNA-histone complexes. As shown in Figure 5B (open bars), cells treated with Hg^{2+} exhibited chromatin fragmentation that increased in a dose-dependent manner.

To investigate whether the potentiation of NMDA-induced Ca^{2+} response by Hg^{2+} also sensitized CGCs to NMDA toxicity, neurons were exposed simultaneously to both Hg^{2+} and sublethal NMDA concentrations for 15 min. As shown in Figure 5A, the combination of 50 μM NMDA and increasing Hg^{2+} concentrations enhanced CGC killing in a dose-dependent manner. NMDA alone, at 50 μM , induced apoptosis in relatively few cells and did not modify MTT metabolism (Figure 5). When CGCs were treated with the combination of 0.5 μM Hg^{2+} and 50 μM NMDA, the formation of both large (Figure 4B, lane 4) and small (Figure 5B) chromatin fragments was enhanced. Higher NMDA levels (up to 500 μM) triggered substantial CGC apoptosis even in the absence of Hg^{2+} as shown by the accumulation of 50 kbp fragments (Figure 4C lane 9). Ca^{2+} channel blockers were effective in preventing also the formation of the 50 kbp fragments induced by 500 μM NMDA alone or by the combination of 500 μM NMDA with Hg^{2+} (Figure 4C lanes 9–16).

Discussion

Perturbation of Ca^{2+} signalling can promote alterations in neurosecretion, affect cell differentiation and plasticity and, eventually, cause cell death, necrosis or apoptosis (Suarez, 1984; Meldolesi and Ceccarelli, 1988; Brown *et al*, 1988; Choi, 1989; Nicotera *et al*, 1994). In a previous study, we showed that exposure of PC12 cells to low Hg^{2+} concentra-

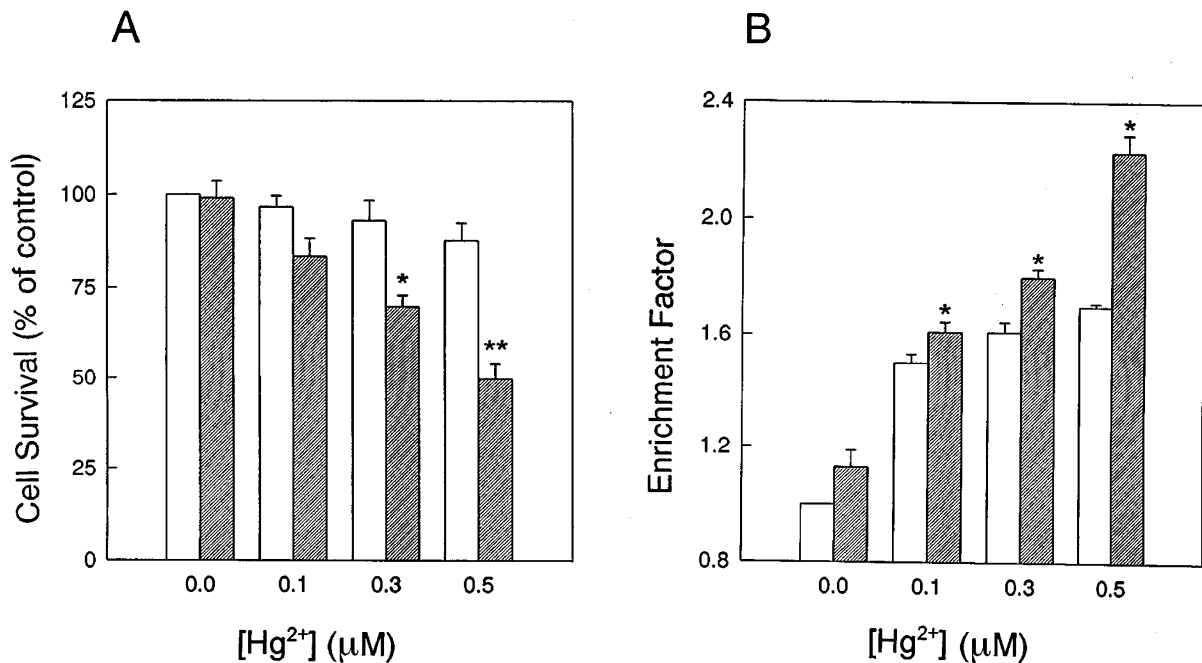


Figure 5 Potentiation of NMDA-induced cell killing by Hg^{2+} . (A) Cells were incubated with different concentrations of Hg^{2+} alone (open bars) or in combination with 50 μM NMDA (shaded bars) for 10 min, and the MTT assay was performed after 24 h. (B) Cells were exposed to Hg^{2+} in the absence (open bars) or in the presence (shaded bars) of 50 μM NMDA. Oligonucleosomal fragmentation was determined by the immunoassay described in Materials and Methods. The enrichment factor was the ratio between the absorbance of the sample and that of the corresponding control and indicates the increase in mono and oligonucleosomal fragments in the lysates obtained from PC12 cells (each point is the mean \pm s.d. of triplicate samples from four separate experiments (* $p < 0.05$ vs. 0.3 μM Hg^{2+} ; ** $p < 0.01$ vs. 0.5 μM Hg^{2+} , by Student's t-test).

tions (0.3–0.5 μM) increased L-type Ca^{2+} channel conductance and potentiated NGF-induced differentiation (Rossi *et al*, 1993). Here we show that treatment of CGCs with the same Hg^{2+} concentrations produced similar alterations in the Ca^{2+} signals elicited by high K^+ , but triggered apoptosis.

$[\text{Ca}^{2+}]_i$ overload elicited either by overstimulation of NMDA channels or by agonists of L-type channels can cause neuronal death (Choi, 1989; Dreyer *et al*, 1990; Lipton, 1994). The latter can take the shape of necrosis or apoptosis often depending on the intensity of the insult (Bonfoco *et al*, 1995; Ankarcona *et al*, 1995). A moderate, but sustained elevation of $[\text{Ca}^{2+}]_i$ can be a very effective stimulus to induce neuronal apoptosis, in particular if it is combined with sensitizing conditions. For example, we have recently shown that nitrosative stress elicits CGC apoptosis by stimulating intrasynaptic release of glutamate receptor agonists, which in turn cause intracellular Ca^{2+} accumulation via NMDA receptor stimulation (Bonfoco *et al*, 1996, Leist M. and Nicotera P. unpublished observations).

Combined activation of both NMDA and L-type channels is often observed during neuronal injury. Enhanced Ca^{2+} entry through L-type channels can stimulate neurosecretion of NMDA agonists that in turn activate postsynaptic receptors. In this study, the protective effect of verapamil, nifedipine and nicardipine that blocked Hg^{2+} -induced Ca^{2+} elevation and Hg^{2+} -induced apoptosis strongly suggest that a direct or indirect activation of L-type channels was involved.

On the other hand, also specific NMDA-channel blockers such as MK-801 prevented apoptosis induced by Hg^{2+} alone or by the combination of Hg^{2+} and NMDA. Increased Ca^{2+} entry through L-type channels is generally better tolerated than that occurring through NMDA receptors (Tymiansky *et al*, 1993). Nevertheless the latter can elicit release of neurotransmitters including glutamate, with a subsequent stimulation of NMDA receptors. In view of these considerations, the following model would explain our findings: Hg^{2+} -induced Ca^{2+} entry through L-type channels stimulates exocytosis of glutamate agonists. The latter in turn trigger further Ca^{2+} influx through NMDA-receptors to reach a sustained, cytotoxic $[\text{Ca}^{2+}]_i$ level. This model is consistent with other observations in our laboratory using donors of nitric oxide as pro-oxidants (Bonfoco *et al*, 1996, Leist M. and Nicotera P. unpublished observations) or with other observations in models of hypoxia (Drejer *et al*, 1985), hypoglycemia (Wieloch, 1985), or various intoxications (Borgetta *et al*, 1990; Ishimaru *et al*, 1992), where release of glutamate plays an important role.

The observation that Hg^{2+} prevented the full recovery of $[\text{Ca}^{2+}]_i$ after NMDA stimulation also suggests that very low NMDA concentrations, which *per se* are not significantly neurotoxic may elicit $[\text{Ca}^{2+}]_i$ increases, sufficient to trigger apoptosis in the presence of Hg^{2+} . In agreement with this interpretation are the findings that: (i) the elevation in $[\text{Ca}^{2+}]_i$ promoted by the combined effect of Hg^{2+} and NMDA was sustained for at least 20 min (ii) the NMDA antagonist, MK-801, prevented Hg^{2+} -induced apoptosis in this system as well as apoptosis elicited by the combination of NMDA and Hg^{2+} . Thus, while the source of NMDA agonists may be autocrine in CGCs treated exclusively with high Hg^{2+}

concentrations, lower levels of the metal would sensitize CGCs to the addition of subtoxic amounts of NMDA.

The downstream events leading to apoptosis in this system are still unclear. Since Ca^{2+} entry leads to activation of the brain nitric oxide synthase (bNOS) with production of nitric oxide (NO), one may expect an involvement of endogenous NO generation in Hg^{2+} -induced CGC apoptosis. However, inhibitors of the nitric oxide synthase (NOS) failed to prevent apoptosis in this system. More likely, direct Ca^{2+} dependent catabolic processes (ie, activation of proteases, mitochondrial dysfunction) are involved here. This possibility is currently being examined.

Morphological studies in organotypic cultures of rat cerebellum have recently shown that non-toxic mercury concentrations are capable of lowering the threshold for glutamate neurotoxicity (Matyja and Albrecht, 1993). Furthermore, low Hg^{2+} concentrations can inhibit glutamate uptake in astrocyte cultures (Brookes, 1992). Thus, a slow release of glutamate at synaptic sites, as shown in other studies (Bonfoco *et al*, 1996; Leist M. and Nicotera P. unpublished observations), in conjunction with a block of glutamate reuptake may lead to a condition of excitotoxicity. To support this hypothesis, recent experiments in our laboratories have shown that exposure of pregnant rats to mercury (0.5 mg/kg/day) results in increased sensitivity to glutamate-induced apoptosis in the CGCs isolated from the pups (Rossi A.D. unpublished observations).

Some obvious differences between undifferentiated PC12 cells and CGCs include the lack of functional NMDA channels in the former and the stage of development. Thus, a sort of autocrine killing involving exocytosis of glutamate-like agonists would not take place in PC12 cells. In addition, it is well known that the implications of enhanced Ca^{2+} signals in mature and immature neurons are quite different. While in immature neuronal cells, increased $[\text{Ca}^{2+}]_i$ following exposure to high K^+ protects cells lacking growth stimulation (Koike *et al*, 1989), Ca^{2+} overload in differentiated neurons rapidly alters neurotransmitter release and can cause cell death (Hartley *et al*, 1993). Ca^{2+} responses to high K^+ are amplified with cell differentiation (Connor *et al*, 1987) and more ion channels are expressed in mature and ageing neurons (Thibault and Landfield, 1996) than in neuroblasts or undifferentiated cell lines (Kalman *et al*, 1990; Janigro *et al*, 1989). According to the ' Ca^{2+} set point' hypothesis (Koike *et al*, 1989) increasing $[\text{Ca}^{2+}]_i$ may be required to prevent the adverse effects of growth factor deprivation in immature neurons, whereas a higher $[\text{Ca}^{2+}]_i$ setpoint would result in killing of mature post-mitotic neurons. Thus, factors downstream to the Ca^{2+} increase may play a relevant role in deciding the response of immature versus differentiated neurons. One such factor regulatory factor may be the level of expression of Ca^{2+} -binding proteins (Masiakowsky and Shooter, 1988).

In conclusion, our findings suggest that subtoxic Hg^{2+} concentrations sensitize CGCs to stimuli that elicits Ca^{2+} signals sufficient to promote apoptosis. The protective effect of MK-801 an NMDA-open channel blocker implies that Ca^{2+} entry through this receptor channel is required to

elicit cell death. This condition would resemble a slow excitotoxic process similar to that putatively involved in chronic neurodegenerative disorders. The mechanism leading to NMDA activation following exposure to Hg^{2+} is under current investigation.

Materials and Methods

Materials

Cell culture media were purchased from GIBCO Laboratories (Grand Island, NY, USA). HgCl_2 , poly-L-lysine, NMDA, fura 2 AM and propidium iodide were obtained from SIGMA, (St. Louis, MO, USA). The cell live/dead kit including calcein AM and etidium homodimer came from Molecular Probes (Eugene, OR, USA), and the cell death detection (ELISA) was provided by Boehringer Mannheim (Germany).

Cell culture

Cultures enriched in granule cells (>95% of the total cell population) were obtained from dissociated cerebella of 7-day-old Sprague-Dawley rats. Cells were isolated according to Schousboe *et al* (1989) and cultured in basal Eagle medium supplemented with 10% FCS, 25 mM KCl, 100 U/ml penicillin, and 0.1 μM streptomycin. Cells were plated on poly-L-lysine-coated dishes or glass coverslips at a density of 3×10^5 per cm^2 . After 48 h culture, 10 μM 1-D-arabinofuranosylcytosine was added to the culture medium to prevent the growth of non-neuronal cells.

Treatment of cultures

Cells were exposed in an incubation buffer composed as follows: 150 mM NaCl, 5 mM KCl, 1.2 mM MgSO_4 , 1.8 mM CaCl_2 , 25 mM HEPES, 10 mM D-glucose, 1.2 mM Na_2HPO_4 . When cells were incubated with NMDA, the buffer was supplemented with 10 μM glycine and Mg^{2+} was omitted to remove the block of the NMDA-receptor (NMDA-R) channel (Scatton, 1993). Before and after exposure, the cells were rinsed three times with incubation buffer and subsequently kept in the initial growth medium to avoid fresh serum toxicity (Schramm *et al*, 1990). To examine the role of extracellular Ca^{2+} influx through L-type or NMDA-R channels, CGCs were pretreated with one of the following L-type channel blockers: verapamil (10 μM), nifedipine (0.5 μM), nicardipine (30 μM), or with the NMDA-R antagonist, MK-801 (1 μM). In some experiments, verapamil was added to the medium after bathing cells in nominally Ca^{2+} -free incubation buffer, to prevent Ca^{2+} influx after medium replacement.

Cell survival

CGC survival was determined by the 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT) (Gerlier and Thomasset, 1986). Cells were cultured in 24-well-plates and the medium was replaced before each time point with a serum-free medium supplemented with 10% (v/v) stock solution (7.5 mg/ml) of MTT. After three hours this medium was replaced with iso-propanol (200 μl) and the absorption was measured in a Labsystem Multiscan MCC/340 ELISA reader with the wavelength set at 590 nm.

Measurement of the $[\text{Ca}^{2+}]_i$

CGCs were cultured on glass coverslips as described above and loaded with 2 μM fura 2-AM (Grynkiewicz *et al*, 1985) for 30 min at

37°C in culture medium. Cells were then rinsed in incubation buffer (as described above) and incubated for 10 min, at room temperature to allow further fura-2 deesterification. Coverslips were inserted into a temperature controlled perfusion chamber (30°C) and placed on the stage of a Zeiss Axiovert microscope equipped with a 100 \times oil immersion Zeiss objective. Perfusion through the chamber was set at approximately 0.5 ml/min. Ca^{2+} changes were measured as the ratio of excitation wavelengths 340/380 with the emission set at 510 nm (29). Ca^{2+} was calculated according the formula: $\text{Ca}^{2+} = K_d (R - R_{\text{min}}) / (R_{\text{max}} - R) (sf_2/sb_2)$ where K_d was assumed to be 180 nM at 30°C, and R was the experimentally determined ratio. R_{max} is the maximal ratio obtained using the Ca^{2+} ionophore, ionomycin, to saturate the dye. MnCl_2 was used to quench the dye and obtain the minimum ratio (R_{min}). sf_2/sb_2 is the ratio of fura-2 fluorescence value at 380 nm for free and Ca^{2+} -bound dye respectively (Grynkiewicz *et al*, 1985). The contribution of extracellular fura-2AM was subtracted after quenching the fluorescence with 50 μM MnCl_2 .

Formation of apoptotic nuclei

CGCs on coverslips were washed and fixed with 80% (v/v) methanol for 15 min at 4°C. After fixation, cells were rinsed, stained for 5 min with 5 $\mu\text{g/ml}$ propidium iodide and examined with a BIO-RAD MRC-600 confocal microscope, equipped with a krypton-argon laser. Nuclei of untreated cells revealed a typical chromatin morphology with distinct organization, whereas apoptotic nuclei were highly fluorescent, condensed, and displayed polarized chromatin aggregates.

DNA fragmentation

The formation of large DNA fragments (ie, 700–50 kbp) was detected by field-inversion gel electrophoresis (FIGE) as previously described (Zhitovskiy, *et al*, 1994). FIGE was carried out using a horizontal gel chamber (HE 100B), a power supply (PS 500 XT), and a Switchback pulse controller, PC 500 from Hoefer Scientific Instruments (San Francisco, CA, USA). Electrophoresis was run at 180 V in 1% agarose gels in 0.5 \times TBE (45 mM Tris, 1.25 mM EDTA, 45 mM boric acid, pH 8.0, at 12°C, with the ramping rate changing from 20 s to 30 s for the first 6 h, 10 s to 20 s for the second 6 h, and 0.8 s to 10 s for the next 12 h, using a forward to reverse ratio of 3 : 1. Calibration of DNA sizes was performed using three sets of pulse markers with overlapping size ranges: chromosomes from *Saccharomyces cerevisiae* (225–2200 kb), 21 successive concatemers of DNA (50–1000 kb), and a mixture of λ DNA Hind III fragments, λ DNA and λ DNA concatemers (0.1–200 kb) purchased from Sigma. DNA was stained with ethidium bromide, visualized using a UV light source (305 nm) and photographed using Polaroid 665 positive/negative film.

Formation of immunoreactive oligonucleosomal fragments was detected using the 'Cell death detection kit, ELISA' obtained from Boehringer-Mannheim. This assay is based on the quantitative sandwich-enzyme immunoassay principle using mouse monoclonal antibodies directed against DNA and histones, respectively. This allows the specific determination of mono- and oligonucleosomes in the 15,000 \times g supernatant fraction of cell lysates.

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